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# MDR1/P-glycoprotein function. II. Effect of hypotonicity and inhibitors on Cl<sup>-</sup> efflux and volume regulation

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**Weaver, James L., Adorjan Aszalos, and Leslie McKinney.** MDR1/P-glycoprotein function. II. Effect of hypotonicity and inhibitors on Cl<sup>-</sup> efflux and volume regulation. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1453-C1460, 1996.—Resistance to anti-tumor drugs can be mediated by overexpression of the multidrug resistance 1 (MDR1) protein (P-glycoprotein). In three MDR1-transfected cell lines (Gill et al. *Cell* 71: 23-32, 1992; Altenberg et al. *Cancer Res.* 54: 618-622, 1994), a hypotonic stress-induced Cl<sup>-</sup> current has been demonstrated that can be inhibited by MDR1 substrates and Cl<sup>-</sup> channel blockers. We tested the hypothesis that MDR1 expression confers additional Cl<sup>-</sup> conductance by measuring regulatory volume decrease (RVD) in four pairs of isogenic cell lines and <sup>36</sup>Cl efflux in two cell lines with and without hypotonic stress. The kinetics of RVD and response to Cl<sup>-</sup> channel blockers were indistinguishable in MDR and parental cells. Additionally, no significant difference was seen between <sup>36</sup>Cl efflux rate constants under hypotonic conditions between NIH/3T3 and L1210 parental and MDR cells. We conclude that, in intact cells, the expression of MDR1 does not alter the rate of volume regulation or the rate of <sup>36</sup>Cl efflux under hypotonic conditions between parental and MDR cells.

multidrug resistance; P-glycoprotein; chloride channel; chloride efflux; cyclosporin; 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; forskolin; 1,9-dideoxyforskolin

CELLS THAT EXPRESS in their membranes the protein called, variously, multidrug resistance 1 (MDR1), P-glycoprotein, or p170 are able to actively extrude cytotoxic drugs and other compounds from their cytoplasm and are said to be multidrug resistant. The MDR1 protein is a member of the ATP-binding cassette family of transport proteins that includes the cystic fibrosis transmembrane conductance regulator (CFTR), which is a Cl<sup>-</sup> channel (20). The MDR1 protein functions as an ATP-dependent pump (12) and can extrude drugs against a concentration gradient (21).

Several recent studies have indicated that this protein may also function as or regulate a Cl<sup>-</sup> conductance. Patch-clamp studies carried out by two different groups have characterized a swelling-activated outwardly rectifying Cl<sup>-</sup> conductance, present in MDR1-transfected NIH/3T3 fibroblasts (7, 23) and BC 19/3 breast cancer cells (1), which was not present in the corresponding parental cell lines. In contrast, studies comparing NIH/3T3 and a colchicine-selected MDR1-expressing derivative (COL1000; Ref. 16) showed that both parental and MDR1-expressing cells have swelling-induced Cl<sup>-</sup> currents. However, the MDR cells exhibited a greater sensitivity to osmotic stress and showed in-

creased anion efflux for a given degree of hypotonicity in the external solution than their parental counterparts. Also, in contrast to the original observations, Ehring et al. (5) carried out patch-clamp experiments on the same transfected NIH/3T3 cell lines used above and observed similar large swelling-induced Cl<sup>-</sup> conductances in both parental and transfected lines. Kunzelmann et al. (14) measured <sup>36</sup>Cl<sup>-</sup> efflux in HT-29 cells and found no difference in the rates of Cl<sup>-</sup> efflux between MDR1-expressing and MDR1-negative cell lines under isotonic or hypotonic conditions. Finally, Rasola et al. (19) measured Cl<sup>-</sup> currents in four MDR1-expressing cell lines and found no association between the amount of MDR1 protein present and the size of the Cl<sup>-</sup> currents.

Given these conflicting reports, we carried out two functional assays of Cl<sup>-</sup> conductance using intact cells to determine whether differences between MDR1-transfected vs. nontransfected lines could be observed. First, the ability of MDR1-expressing vs. parental cells to carry out a regulatory volume decrease (RVD) after osmotic stress was measured. Hypotonic swelling activates various forms of ion transport, leading to net loss of ions with concomitant loss of water and a return to normal cell size (hence the term RVD). If it is assumed, by analogy to a number of other cell types (see Refs. 4 and 9 for reviews), that a volume-activated Cl<sup>-</sup> current is necessary for RVD, and then MDR1-expressing cells might show an increased rate of RVD compared with parental cells if they also express additional Cl<sup>-</sup> conductance. Experiments were carried out in various combinations of four parental cell lines and a stable MDR1-transfected derivative of each line: NIH/3T3 and NIH/3T3MDR murine fibroblasts, FEM-X and FEM-XvMDR human melanoma cells, and two murine lymphomas, L1210, L1210vMDR and L5178Y, L5178YvMDR.

Second, the release of <sup>36</sup>Cl<sup>-</sup> from two pairs of cell lines under isotonic and hypotonic conditions was compared to determine whether MDR1-transfected cells would show increased rates of Cl<sup>-</sup> efflux after cell swelling.

No difference in the regulatory volume response between the parental and MDR cell lines was found. Consistent with these results, no significant differences in the rate constants for swelling-induced Cl<sup>-</sup> efflux between the MDR1-transfected and parental lines of two cell types, NIH/3T3 and L1210, were found. A small but significant difference in the rate constant for Cl<sup>-</sup> efflux was noted under isotonic conditions for NIH/3T3 but not L1210 cells.

## MATERIALS AND METHODS

**Growth conditions, inhibitors, and reagents.** The cell lines, drugs, and solutions used for these experiments are described in the accompanying study (23a).

**$\text{Cl}^-$  efflux measurements.**  $\text{Cl}^-$  efflux was measured using  $\text{Na}^{36}\text{Cl}$  (Amersham, Arlington Heights, IL). NIH/3T3 parental or MDR cells were plated in 24-well plates at  $4 \times 10^5$ /well and allowed to settle overnight. Two wells of either parental or MDR cells were labeled by incubation in Dulbecco's modified Eagle's medium (DMEM) with 20  $\mu\text{Ci}/\text{ml}$   $^{36}\text{Cl}^-$  for 90 min at  $37^\circ\text{C}$ . Cells were washed three times with  $\text{Na}^+$  Hanks'-glucose solution plus or minus drugs. Next, 0.5 ml of  $\text{Na}^+$  Hanks'-glucose solution was added to each well. Every 2 min, this buffer was removed to a scintillation vial and fresh buffer was added. After 16 min, one well was switched to hypotonic buffer [50%  $\text{Na}^+$  Hanks'-glucose (drugs), 50% water]. At the 28-min time point, the cells were lysed with 0.5 ml of 5% Triton X-100 and the wells were scraped, and all cells and supernatant were removed to a scintillation vial. The counts per minute (cpm) of  $^{36}\text{Cl}^-$  remaining in the cell layer at each time point were calculated, and the data were normalized and represented as the fraction of the total cpm remaining in the cells. For the 8- to 16-min (isotonic and hypotonic) and 18- to 30-min (hypotonic only) time points, the data were fitted to this single exponential equation using the program NFIT (University of Texas Medical Branch, Galveston, TX)

$$y = A + B \times \exp(-x/T)$$

where  $A$  is background,  $B$  is starting value minus background, and  $T$  is the time constant. Efflux rate constants were calculated as  $1/T$ , and means  $\pm$  SE were obtained for each experiment. Statistical significance was calculated using Student's  $t$ -test. All calculations were performed using Quattro Pro for Windows version 5.0 (Borland, Scotts Valley, CA).

For the nonadherent L1210 and L1210vMDR cells, methods were adapted from Grinstein et al. (8). For each run,  $\sim 100 \times 10^6$  cells were washed once and resuspended in DMEM with 45  $\mu\text{Ci}/\text{ml}$   $\text{Na}^{36}\text{Cl}$ . After 60 min of incubation, the cells were quickly washed twice and resuspended in two tubes in DMEM to a concentration of  $10 \times 10^6/\text{ml}$ . Aliquots of 100  $\mu\text{l}$  were taken at the start and at 1-min intervals for 10 min. Each aliquot was centrifuged through a mixture of 10 parts dibutyl phthalate and 3 parts corn oil. The supernatant was removed to one scintillation vial and the pellet resuspended in 0.5 ml of 5% Triton X-100 and transferred to a separate scintillation vial. After 4 min, an equal volume of water or DMEM was added to the hypotonic or isotonic tubes, respectively. Data from 10-min counts were expressed as the normalized fraction of cpm in the cell pellet. Data were fitted by a single exponential, using the 1- to 4-min or 1- to 10-min data points for isotonic and 5- to 10-min data points for hypotonic data. Rate constants were calculated as described above for the NIH/3T3 cells.

**Measurement of membrane potential.** Membrane potential was measured by flow cytometry using the positively charged cyanine dye DiOC(6)<sub>3</sub> (Molecular Probes, Eugene, OR) using the method of Wilson et al. (26).

**Measurements of cell volume.** Most cell volume measurements were obtained on a Coulter ZM cytometer connected to a Zenith 286 computer containing a PCA II interface board from The Nucleus, Oak Ridge, TN. Volume data were collected on  $\sim 1 \times 10^4$  cells and mean volume calculated using commercial software written for the PCA II interface. Cells were suspended at  $\sim 2 \times 10^6/\text{ml}$  in  $\text{Na}^+$  Hanks' buffer with 5 mM glucose unless otherwise noted and were equilibrated for 10 min (L1210, L5178, FEM-X) or 30 min (NIH/3T3). The baseline was established by measuring the mean volume of

control cells in triplicate every 10 min. A test sample was diluted 50% with water and volume measurements taken at 1- to 3-min intervals. The results are expressed normalized to the initial control volume, which varied by  $<5\%$  over 30 min.

In some experiments on the lymphoid cell lines, changes in cell volume were estimated by quantitating changes in forward angle light scatter (FS) over time. This is a variation on the system that McManus et al. (17) used to measure cell volume regulation. Changes in light scatter were measured by flow cytometry on an Epics Elite cell sorter (Coulter, Hialeah, FL) equipped with a Time Zero module (Cytek, Fremont, CA). Cells were resuspended in the indicated buffers at a concentration of  $\sim 2 \times 10^6/\text{ml}$ . Data were collected for 5 min to establish a baseline, then water was added to dilute the cell suspension by 50%. Data were collected for an additional 20 min and analyzed using Multitime software, version 2.5 (Phoenix Flow Systems, San Diego, CA). The data were automatically binned into 64 groups so that each data point represents the average for 23.4 s.

Figure 1 shows a comparison of the FS and Coulter cytometer data in L5178Y cells as they respond to hypotonic stress. Coulter volume measurements show that, as expected, the cells initially swell and then regulate back toward their original volume. Changes in the FS signal occur on a similar time scale but in the opposite direction. After addition of water, the FS signal, which usually increases with particle size, instead drops abruptly before returning toward baseline and seems to show that the cells are shrinking in response to hypotonic stress. A possible explanation for this apparently anomalous change in FS has to do with the surface morphology of lymphoid cells. Grinstein et al. (10) have observed by scanning electron microscopy that the surface of resting peripheral blood lymphocytes is covered with many fingerlike processes. After exposure to hypotonic stress the cell expands and the "fingers" are diminished in size relative to the expanded cell volume. As the cells return toward their original size during RVD, the villi return to their original size. This type of change in cell surface morphology after hypotonic stress has also been observed in Erlich ascites cells (11). The resting cell has a very rough surface that is effective at light scatter, and the swollen cell has a much smoother surface that is less likely to scatter light. Thus the forward light scatter signal was inversely correlated with changes in cell volume but only in L1210 and L5178 cells. In NIH/3T3 and FEM-X cells, the observed changes in FS were too small to be practically useful.

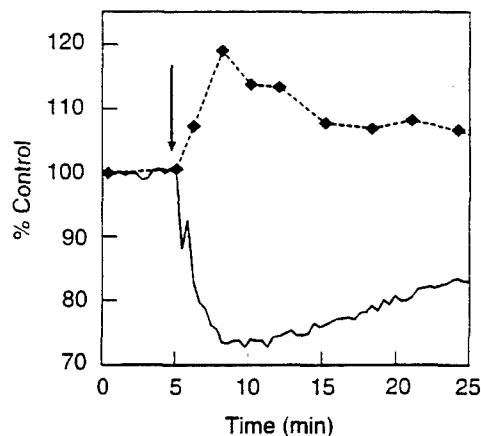


Fig. 1. Kinetics of forward scatter and Coulter volume changes in response to hypotonic stress in L5178Y cells. Solid line, forward angle light scatter (FS) signal; dashed line, Coulter volume response. Arrow, dilution with 50%  $\text{H}_2\text{O}$ .

## RESULTS

**RVD in MDR and parental cells.** Cells that are exposed to hypotonic stress swell and then undergo an RVD to recover their original volume. In many cell types, RVD involves the activation of  $\text{K}^+$  and  $\text{Cl}^-$  conductances, leading to net efflux of  $\text{KCl}$  and the passive loss of water (9). If the MDR1 protein functions as or regulates a volume-activated  $\text{Cl}^-$  channel, then RVD could be faster in MDR cells, assuming that the induced  $\text{Cl}^-$  conductance is a significant fraction of the total cellular  $\text{Cl}^-$  conductance and is rate limiting for volume regulation. Figure 2 shows the time course of the RVD in parental and MDR cells using four pairs of cell lines. Cells were equilibrated in NaCl Hanks' solution and then diluted to 50% with water. Coulter volume measurements showed that cells swelled to 120–130% of their initial volume within ~5 min and recovered within 20–30 min. In all four cell lines, there was no significant difference in the rate of RVD between parental and MDR1-transfected cells. Although the FS measurements are continuously acquired, we verified their accuracy for the initial stage of volume regulation by taking frequent Coulter volume measurements (10- to 12-s intervals for 5–7 min) on the L1210 cell lines. This experiment showed no difference in the rates of RVD ( $n = 3$ ). The MDR cells showed slightly greater initial swelling, which is the opposite of what would be expected if the MDR cells had a significantly greater  $\text{Cl}^-$  conductance (data not shown).

Resting volumes for each cell line were very consistent on a given day (<5% variability) but showed greater variation across days. Values for NIH/3T3 and FEM-X cells ranged from ~1,600 to 2,800 fl and for L1210 and L5178Y ranged from 470 to 830 fl. No consistent differences in resting volume were noted between parental and MDR cells from any of these cell lines.

*The rate of RVD is not increased by increasing cation conductance.* Given the lack of difference in the rates of

RVD between parental and MDR1-transfected cells, several assumptions underlying the RVD experiments were more directly examined. Was it possible that  $\text{Cl}^-$  conductance was not rate limiting for RVD? To address this question, cells were exposed to the  $\text{K}^+$  ionophore valinomycin to artificially increase  $\text{K}^+$  permeability. The effectiveness of valinomycin in altering  $\text{K}^+$  permeability was confirmed using the membrane potential-sensitive cyanine dye DiOC(6)<sub>3</sub> to show that 1  $\mu\text{M}$  valinomycin caused hyperpolarization of L5178Y and L1210 cells in 4.5 mM  $\text{K}^+$  Hanks' solution (data not shown). Even if  $\text{K}^+$  is not the normal cation used in RVD, the increased permeability to  $\text{K}^+$  should still increase the rate of RVD if cation conductivity is rate limiting. In parental L1210 and L5178Y cells, the rates of RVD in control and valinomycin-treated cells were found to be similar (Fig. 3A). Increasing valinomycin concentration to 20  $\mu\text{M}$  caused no alteration in the rate of RVD (data not shown). Similar experiments were not carried out on MDR cells because valinomycin is a substrate for the MDR1 protein (15, 22, 25) and results would not be interpretable.

Because valinomycin had no effect on RVD, one experiment was carried out to determine whether RVD was discernibly sensitive to the gradient for  $\text{K}^+$  and whether parental and MDR cells responded similarly to altered  $\text{K}^+$  gradients. Cells were exposed to high- $\text{K}^+$  Hanks' solution and then subjected to hypotonic stress (Fig. 3B). Under these conditions, the driving force for  $\text{K}^+$  is inward, and, if  $\text{K}^+$  conductance is sufficiently large, then  $\text{K}^+$  will go into the cell accompanied by anions and water and produce a second phase of swelling after the initial response to hypotonicity. The data show that cells placed in high- $\text{K}^+$  solution initially swelled to ~120% of resting volume after hypotonic stress. Three of the four parental cell lines then underwent a secondary swelling over the next 30 min (Fig. 3B) to a maximum of 150%. Secondary swelling was also observed in MDR1-transfected L1210 (Fig. 3C)

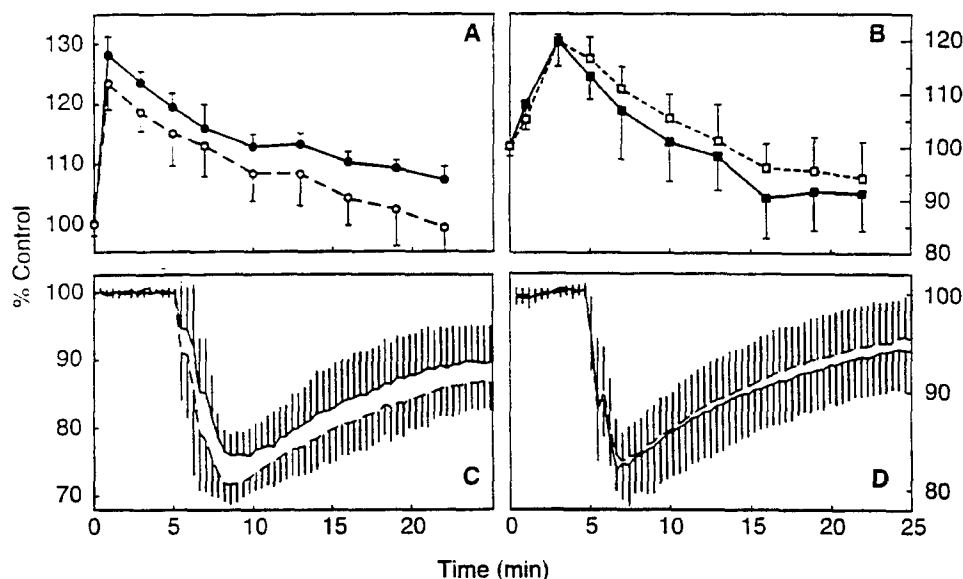


Fig. 2. Regulatory volume decrease (RVD) response in parental and multi-drug resistant (MDR) cell lines. All values are means of 4–8 experiments; error bars represent SD. A: changes in Coulter volume with 50%  $\text{H}_2\text{O}$  at 0 min ( $n = 4$ ). Solid line, NIH/3T3; dashed line, NIH/3T3MDR. B: changes in Coulter volume with 50% dilution with  $\text{H}_2\text{O}$  at 0 min ( $n = 4$ ). Solid line, FEM-X; dashed line, FEM-XvMDR. C: changes in FS with 50% dilution with  $\text{H}_2\text{O}$  at 5 min ( $n = 5$ ). Solid line, L5178Y; dashed line, L5178YvMDR. D: changes in FS with 50% dilution with  $\text{H}_2\text{O}$  at 5 min ( $n = 8$ ). Solid line, L1210; dashed line, L1210vMDR.

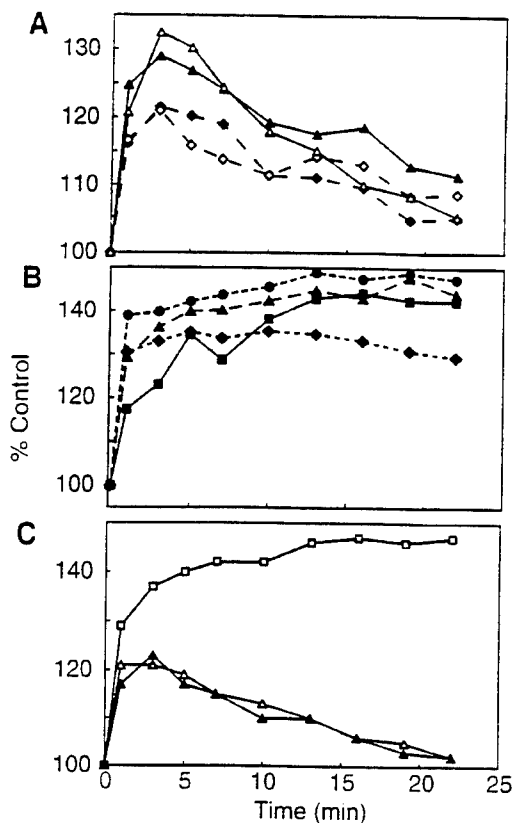


Fig. 3. Effect of various treatments affecting  $\text{K}^+$  flux on RVD measured by Coulter volume. A: RVD with valinomycin. Solid lines, L1210; dashed lines, L5178Y;  $\blacksquare$  or  $\blacktriangle$ , no drug;  $\square$  or  $\triangle$ , 1  $\mu\text{M}$  valinomycin at -5 min. B: RVD in 140 mM  $\text{K}^+$  Hanks'-glucose solution. Solid line, FEMX; dashed line/triangles, L1210; dashed line/circles, NIH/3T3; dashed line/diamonds, L5178Y. C: RVD of L1210vMDR cells.  $\blacktriangle$ ,  $\text{Na}^+$  Hanks'-glucose solution;  $\triangle$ , 0 mM  $\text{K}^+$  in  $\text{Na}^+$  Hanks'-glucose solution;  $\square$ , 140 mM  $\text{K}^+$  Hanks'-glucose solution.

and NIH/3T3 cells (maximum = 140%; data not shown). These data indicate that volume regulation can be altered by the direction of the driving force for  $\text{K}^+$  in both parental and MDR cells but do not prove that  $\text{K}^+$  is necessarily the primary cation involved in volume regulation.

**$\text{Cl}^-$  channel blockers and RVD.** An alternative approach to testing whether an MDR1-associated  $\text{Cl}^-$

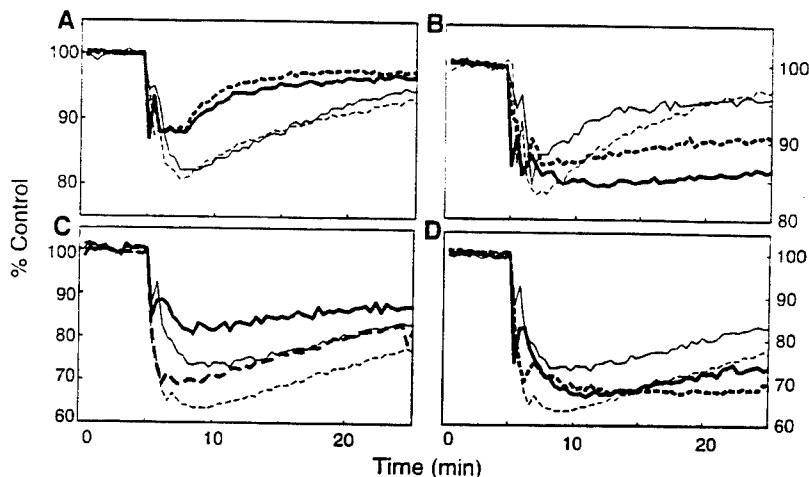
channel participates in RVD was to try to block RVD using compounds known to block the volume-activated  $\text{Cl}^-$  current in MDR1-transfected NIH/3T3 cells: 1,9-dideoxyforskolin (DDFSK; effective at 50  $\mu\text{M}$ ), forskolin (FSK; 50  $\mu\text{M}$ ), and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS; 100  $\mu\text{M}$ ) (6, 23). In NIH/3T3 and FEM-X cells, 50  $\mu\text{M}$  DDFSK produced some slowing of RVD, but the amount of inhibition was similar in parental and MDR1-transfected cells (data not shown). A complete block of RVD in both NIH/3T3 and NIH/3T3MDR cells resulted from treatment with 100  $\mu\text{M}$  DDFSK (data not shown). In contrast, DDFSK had no effect on RVD in either parental or MDR1-transfected L1210 cells. No effect on RVD was seen in L1210 cells with FSK at a higher concentration of 150  $\mu\text{M}$ . DIDS was tested in both L1210 and L5178Y cells, at 500 and 150  $\mu\text{M}$ , respectively (Fig. 4, A and C). Although DIDS reduced the extent of initial swelling in each cell line, there was no differential effect on the MDR cells compared with parental cells. The rates of recovery were also not noticeably affected. In the L5178Y cell lines, DIDS was tested at concentrations between 50 and 700  $\mu\text{M}$ , with no differential effect on RVD observed (data not shown).

As a positive control, we tested the effect of the  $\text{Cl}^-$  channel blocker 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) on RVD. Figure 4, B and D, shows that, in L1210 and L5178Y cells, 700 or 1,000  $\mu\text{M}$  SITS inhibited RVD. In addition, Coulter volume data showed that 700  $\mu\text{M}$  SITS blocked RVD in both NIH/3T3 cell lines (data not shown). Finally, the  $\text{Cl}^-$  channel blockers niflumic acid (90  $\mu\text{M}$ , L1210) and pentylene-tetrazole (2 mM, L1210) were tested for their effects on RVD and had no differential effect.

Cyclosporin A (CsA), at a concentration that completely inhibits rhodamine 123 (R123) exclusion (0.8  $\mu\text{M}$ ), was tested for its affect on RVD. In L5178Y or L5178YvMDR cells this MDR1 inhibitor had no effect on the kinetics of RVD (data not shown).

**$\text{Cl}^-$  efflux measurement in NIH/3T3 and NIH/3T3MDR cells.** Given the lack of difference in RVD between parental and MDR cell lines, a series of  $\text{Cl}^-$  efflux experiments was carried out to directly measure

Fig. 4. Effect of SITS or DIDS on volume regulation by FS. A: DIDS and L1210 cells. Solid lines, L1210. Dashed lines, L1210vMDR. Light lines, no drug; heavy lines, 500  $\mu\text{M}$  DIDS. Cells were exposed 30 min before data collection. B: SITS and L1210 cells. Same line scheme as A, except that cells were exposed to 700  $\mu\text{M}$  SITS 10 min before data collection. C: DIDS and L5178Y cells. Solid lines, L5178Y; dashed lines, L5178YvMDR. Light lines, no drug. Heavy lines, 150  $\mu\text{M}$  DIDS at 30 min before data collection. D: SITS and L5178Y cells. Same line scheme as C, except that cells were exposed to 1 mM SITS 10 min before data collection.



the changes in  $\text{Cl}^-$  permeability after hypotonic stress. The time course of efflux of  $^{36}\text{Cl}^-$  from NIH/3T3 parental and NIH/3T3MDR cell lines under isotonic and hypotonic conditions was measured. Data from a representative experiment are shown in Fig. 5, expressed as the fraction of labeled  $\text{Cl}^-$  remaining in the cells with time. Under isotonic conditions, after an initial period of rapid  $^{36}\text{Cl}^-$  efflux (0–6 min), the efflux rate became monoexponential. Dilution of the bathing medium by 50% (at ~17 min) led to an increase in the rate of  $^{36}\text{Cl}^-$  efflux, as would be expected if cell swelling activated a  $\text{Cl}^-$  conductance.

Efflux rate constants, calculated as described in MATERIALS AND METHODS and averaged for five experiments, are shown in Fig. 6A for each cell line before and after the dilution of the medium. No significant difference in the efflux rate constant was observed between parental and MDR cell lines under hypotonic conditions. Under isotonic conditions, a small but statistically significant increase in the efflux rate constant was noted in MDR vs. parental cells. In one experiment, as a point of reference, fractional  $\text{Cl}^-$  efflux was converted to a true efflux rate by normalizing cpm values to specific activity, cell number, and cell volume (obtained from Coulter counter measurements). Values for parental NIH/3T3 cells ranged from 1.79  $\text{mmol} \cdot \text{l}^{-1} \cdot \text{cell}^{-1}$

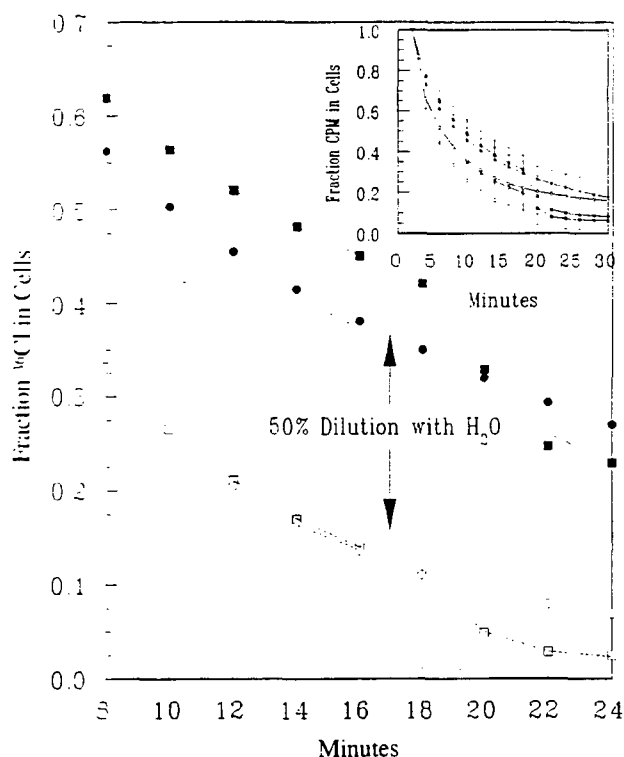


Fig. 5. Representative data of efflux of  $^{36}\text{Cl}^-$  from NIH/3T3 and NIH/3T3MDR cells. Values are normalized counts/min (cpm) of  $^{36}\text{Cl}^-$  remaining in the cells at each time point. ●, NIH/3T3 isotonic conditions; ■, NIH/3T3 with 50% hypotonicity after 16-min time point; ○, NIH/3T3MDR isotonic conditions; □, NIH/3T3MDR 50% hypotonicity after 16-min time point. Solid lines, fitted curves for parental data; dashed lines, fitted curves for MDR data. Inset: averaged data ( $n = 5$  experiments) for entire time period; error bars represent SE.

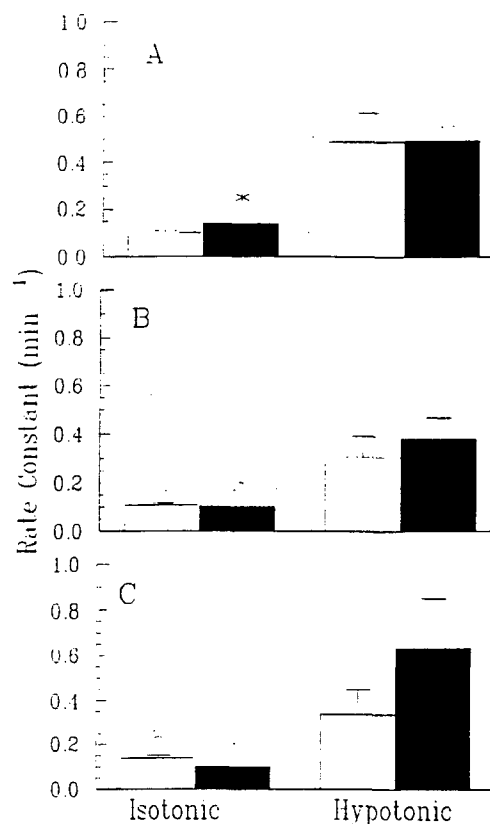


Fig. 6. Values of fitted efflux rates for NIH/3T3 (open bars) and NIH/3T3MDR (solid bars)  $^{36}\text{Cl}^-$  efflux data. Because isotonic/hypotonic runs were paired, for each measurement of the hypotonic rate constant, two rate constants for isotonic conditions were obtained. A: no drug treatment: isotonic mean,  $n = 10$ ; hypotonic mean,  $n = 5$ . B: 5  $\mu\text{M}$  valinomycin: isotonic mean,  $n = 8$ ; hypotonic mean,  $n = 4$ . C: 50  $\mu\text{M}$  bumetanide: isotonic mean,  $n = 8$ ; hypotonic mean,  $n = 4$ . Error bars indicate SE. \*Significant differences (at  $P < 0.05$ ) between parental and MDR; ☆ significant differences between drug treated (B or C) and no drug (A).

$\text{water}^{-1} \cdot \text{min}^{-1}$  under isotonic conditions to 3.33  $\text{mmol} \cdot \text{l}^{-1} \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$  under hypotonic conditions.

Because it was possible that, under hypotonic conditions, anion permeability was not rate limiting and an MDR-associated  $\text{Cl}^-$  conductance was masked, efflux experiments were repeated in the presence of 5  $\mu\text{M}$  valinomycin. No significant difference in rate constants between parental and MDR cells under isotonic or hypotonic conditions was observed after valinomycin treatment ( $n = 4$ , Fig. 6B). Likewise, there was no significant difference between rate constants of untreated vs. valinomycin-treated cells under hypotonic conditions for either parental or MDR cell lines. Under isotonic conditions, there was no significant change in the rate constant for parental cell lines, but the rate constant for valinomycin-treated MDR cells showed a slight but significant decline. Together, these data indicate that cation permeability was not rate limiting for  $\text{Cl}^-$  efflux.

Last, to determine whether another  $\text{Cl}^-$  transport system might be masking an MDR-associated  $\text{Cl}^-$  pathway, efflux experiments were repeated in the presence of bumetanide, an inhibitor of the  $\text{Na}^+ \cdot \text{K}^+ \cdot \text{Cl}^-$  cotransporter. A concentration of 50  $\mu\text{M}$  was used that

has been shown to effectively block activity of the transporter without blocking other  $\text{Cl}^-$  efflux pathways (18). No significant difference in the rate constants between parental and MDR cells under isotonic or hypotonic conditions was revealed by this treatment (Fig. 6C;  $n = 4$ ). Small but significant increases in the rate constants for both parental and MDR cell lines were observed in bumetanide-treated compared with untreated control, but only for the isotonic condition.

**$\text{Cl}^-$  efflux measurements in L1210 and L1210vMDR cells.** Efflux measurements were also performed on the L1210 and L1210vMDR cell lines. Representative efflux data are shown in Fig. 7 and show the same pattern of increased  $\text{Cl}^-$  efflux after hypotonic stress as the NIH/3T3 cells. Comparison of efflux rate constants (Fig. 8A) indicates that there was no significant difference between parental and MDR cells under isotonic or hypotonic conditions ( $n = 3$ ). Addition of 5  $\mu\text{M}$  valinomycin did not significantly affect the rate constants of  $^{36}\text{Cl}^-$  efflux in either cell type ( $n = 2$ , Fig. 8B).

## DISCUSSION

The hypothesis that expression of MDR1 protein is associated with increased  $\text{Cl}^-$  conductance was tested by measuring RVD and  $^{36}\text{Cl}^-$  efflux in parental and MDR1-transfected cell lines. After hypotonic stress, parental and MDR1-transfected cells underwent RVD with similar kinetics (Fig. 2). However, since none of these cell lines approached their predicted van t'Hoff volumes after exposure to hypotonic solution, it was possible that the MDR-associated  $\text{Cl}^-$  conductance was only transiently activated and affected only the early

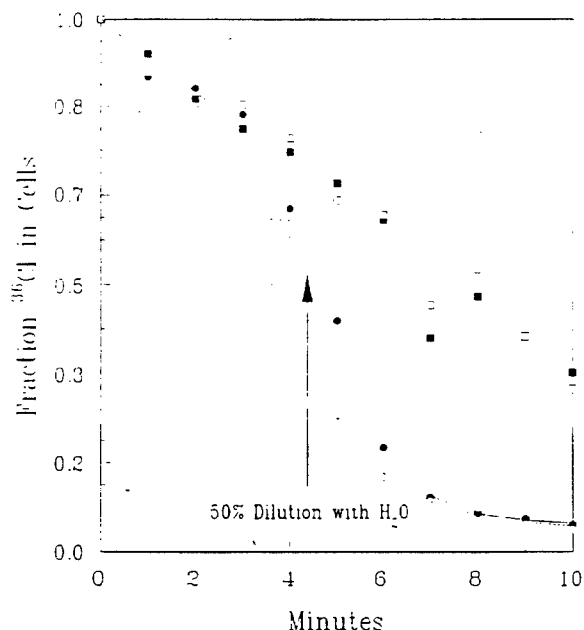


Fig. 7. Representative data of efflux of  $^{36}\text{Cl}^-$  from L1210 and L1210vMDR cells. Values are normalized cpm of  $^{36}\text{Cl}^-$  remaining in the cells at each time point. ■, L1210 isotonic conditions; ●, L1210 with 50% hypotonicity after 4-min time point; □, L1210vMDR isotonic conditions; ○, L1210vMDR 50% hypotonicity after 4-min time point. Solid lines, fitted curves for parental data; dashed lines, fitted curves for MDR data.

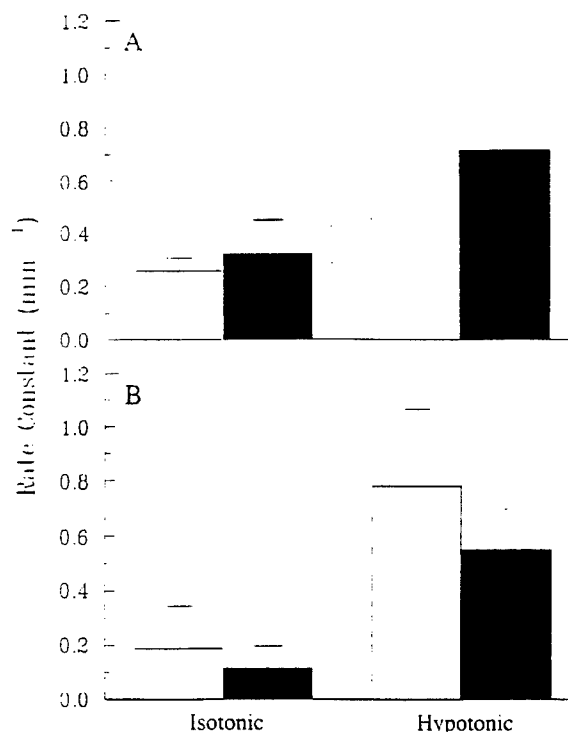


Fig. 8. Values of fitted efflux rates for L1210 (open bars) and L1210vMDR (solid bars)  $^{36}\text{Cl}^-$  efflux data. A: no drug treatment. B: 5  $\mu\text{M}$  valinomycin. Differences between parental and MDR or no drug and valinomycin were not significantly different at  $P < 0.05$ .

phase of the RVD process. However, rapid measurements of RVD over the first 5 min with the L1210 cell lines showed that no previously undetected RVD was occurring in the MDR cell line. Data from NIH/3T3 lines were more variable but did not reliably show large early differences in RVD.

It is unlikely that the lack of observed difference in the rate of RVD between parental and MDR1-expressing cells was due to absent or insufficient (i.e., rate-limiting) cation conductive pathways. Parental cells exposed to the  $\text{K}^+$  ionophore valinomycin did not show an increased rate of RVD in hypotonic NaCl Hanks' solution compared with untreated controls even at high (20  $\mu\text{M}$ ) doses of ionophore. In addition, experiments carried out in high- $\text{K}^+$  solutions showed that volume regulation in both parental and MDR1 cells could be altered in a similar manner by changing the direction of the  $\text{K}^+$  gradient (Fig. 3).

Attempts to unmask an MDR1-associated  $\text{Cl}^-$  conductance using reported blockers of MDR1-associated  $\text{Cl}^-$  current were also unsuccessful. Neither DDFS, FSK, nor DIDS produced differential inhibition of RVD in any of the cell lines tested, although partial inhibition of RVD in paired MDR and parental cell lines was seen (Fig. 4 and see text). This partial inhibition may have been due to other nonspecific actions of these blockers. For example, FSK blocks "n" type  $\text{K}^+$  channels in T lymphocytes that are known to participate in volume regulation (13). In contrast, SITS, a  $\text{Cl}^-$  channel blocker known to inhibit RVD in other cell types, also blocked RVD in the cell lines used here. In agreement with our observations, it has been shown recently (5) that, in the



NIH/3T3 parental and MDR cell lines, DIDS and 5-nitro-2-(3-phenylpropylamino)benzoic acid block volume-activated  $\text{Cl}^-$  channels to a similar extent in both parental and MDR lines.

To more directly measure the effect of MDR1 expression on  $\text{Cl}^-$  permeability,  $^{36}\text{Cl}$  efflux was evaluated in two pairs of cell lines, NIH/3T3, NIH/3T3MDR and L1210, L1210vMDR. The results showed no significant difference in  $^{36}\text{Cl}$  efflux rate constants in NIH/3T3MDR or L1210vMDR compared with parental cells under hypotonic conditions (Figs. 6 and 8). A small but significant difference in the isotonic rate constant between parental and MDR cells was observed in NIH/3T3 but not L1210 cells. Neither basal nor hypotonic-induced rates of  $^{36}\text{Cl}$  efflux were increased by valinomycin (Figs. 6B, 8B). Although this indicates that cation conductance was not rate limiting for  $^{36}\text{Cl}^-$  efflux, this interpretation is complicated by the fact that valinomycin is also an inhibitor of MDR1 transport function (5a, 25). However, since treatment with CsA also had no effect on  $^{36}\text{Cl}$  efflux (data not shown), it appears that competitive inhibition of MDR1 transport does not alter the availability of  $\text{Cl}^-$  efflux pathways. Confirming this observation, treatment with CsA had no effect on RVD in the L5178Y and L5187YvMDR cell lines at a concentration that blocks R123 efflux completely (data not shown).

Our data support the findings of Ehring et al. (5) who, with the use of the same MDR1-transfected NIH/3T3 cells that were used by us and by Valverde et al. (23), found no difference in the levels of swelling-induced  $\text{Cl}^-$  conductance. Our findings differ from those of Luckie et al. (16) who found a differential anion efflux between parental and colchicine-selected NIH/3T3 cells at levels of osmotic stress >30% dilution.

Given the lack of difference between parental and transfected cells, was it possible that there were simply too few MDR1-associated  $\text{Cl}^-$  channels to significantly increase total cellular  $\text{Cl}^-$  conductance relative to parental cells? The data from the accompanying study (23a) indicate that the number of MDR1-binding sites per cell is 8,000–55,000. Several estimates of the number of  $\text{Cl}^-$  channels necessary to account for  $\text{Cl}^-$  efflux (3) or current (27) observed during RVD range from 350 to 1,000 channels per cell. Thus, in all cell lines tested, the numbers of MDR1 antibody binding sites per cell are significantly in excess of the number of  $\text{Cl}^-$  channels per cell likely to be needed for all of the  $\text{Cl}^-$  channel activity needed to carry out RVD. The lack of effect of valinomycin on both RVD and  $^{36}\text{Cl}$  efflux shows that cation permeability is not likely to be rate limiting.

In summary, we have found that RVD and  $^{36}\text{Cl}$  efflux rate constants were not different in several lines of parental vs. MDR1-expressing cells. Drugs reported to inhibit MDR1-associated  $\text{Cl}^-$  channel activity were not able to differentially affect RVD in parental vs. MDR cells. If the MDR1 protein is associated with a volume-activated  $\text{Cl}^-$  transport pathway, it is not detectable in intact cells using these functional assays.

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Portions of this work have been previously published in abstract form (24).

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